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PRINCIPAL INVESTIGATOR: Michael R. Freeman, PhD

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
Los Angeles, CA 90048

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14. ABSTRACT Epidemiologica I data indicate that cholesterol-lowering pharmacotherapy, primarily HGM-CoA-reductase inhibitors ("status"), reduce the risk of aggressive prostate cancer (PCa). The FDA-approved anti obesity drug, Orlistat, which inhibits the enzyme fatty acid synthase (FASN), has been shown to slow the growth of human prostate tumors in mice. Despite these advances, studies of lipid metabolism in PCa have lagged behind other areas of research on cell signaling, and limited information is available about how these promising preclinical and clinical data might be leveraged to improve patient outcomes. Our hypothesis is that PCa progression is dependent on a palmitoyl-protein network regulated by FASN. We predict that the activity of this network can be suppressed by reducing levels of circulating cholesterol. Specific Aims: We will challenge this hypothesis with the following specific A; Identify critical palmitoyl-proteins in the FASN subnetwork.					
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Introduction

Prostate cancer (PCa) is a leading cause of cancer deaths in the United States. Although remarkable progress has been made in the treatment of late-stage PCa, this malignant disease still kills about 27,540 American men annually.¹ Thus, novel therapies are urgently needed to prevent or delay PCa progression to end stage. Compared with normal prostate cells, an important feature of PCa cells is significantly increased *de novo* synthesis of fatty acids and cholesterol, mainly caused by increased expression of fatty acid synthase (FASN) and several other lipogenic enzymes.² Notably, FASN expression correlates with PCa progression, reaching its apex in hormone-insensitive bone metastases.³ Functional studies suggested that FASN promotes PCa progression partly via suppressing apoptosis.⁴ In addition, inhibiting FASN with Orlistat, an FDA-approved anti-obesity drug, suppresses the growth of human prostate tumors in nude mice.⁵ Despite these advances, the role of lipid metabolism in PCa progression remains poorly understood. This lack of knowledge hinders the clinical use of lipid-modifying agents, because critical information on targets and pathways is not yet available.

In human cells, FASN catalyzes the synthesis of 16-carbon palmitate, a substrate for protein palmitoylation, a type of post-translational modification more accurately known as fatty *S*-acylation. In palmitoylation, long-chain fatty acids are attached to specific cysteine residues of proteins via labile thioester bonds.⁶ Palmitoylation is the only reversible lipid modification, regulating the shuttling of proteins between subcellular compartments and modulating protein activity, stability, and complex formation.⁷ Due to the saturated nature of palmitate, palmitoylation preferentially targets a subset of proteins to cholesterol-rich membrane domains known as lipid rafts.⁸ Accumulating evidence has suggested that circulating and membrane cholesterol promotes PCa progression and that lowering cholesterol via statins reduce the risk of aggressive PCa.⁹

Taken together, the epidemiological, experimental, and clinical observations led to our hypothesis that ***PCa progression is dependent on a palmitoyl-protein network regulated by FASN*** and that ***the activity of this network can be suppressed by pharmacologic reduction of circulating cholesterol level***. In this DoD-funded project, we applied a mass spectrometry (MS)-based quantitative palmitoyl-proteomics method, which we developed and further improved, to identify critical nodes and network relationships in the FASN-dependent palmitoyl-protein network in PCa cells. We also tested *in vivo* whether this network is vulnerable to an intervention that employs a dietary strategy in combination with an FDA-approved cholesterol-lowering drug, ezetimibe.

Keywords

Caveolin-1, Cholesterol, Fatty acid synthase, Lipid rafts, Mass Spectrometry, Metastasis, Palmitoylation, PalmPISC, Proteomics, SILAC

Overall Project Summary

Specific Aim 1: Identify critical palmitoyl-proteins in the FASN subnetwork. Test their functional roles.

Aim 1, Task 1. Identify the palmitoylation sites on the AR and palmitoylated proteins that associate with AR and FASN.

Proposed plan: We will use a novel proteomic strategy developed in our laboratory, termed Palmitoyl-protein Identification and Site Characterization (PalmPISC),⁸ to verify the single known palmitoylation site on the AR, identify any other palmitoylation sites on the protein, and identify novel palmitoylation proteins and palmitoylated sites on proteins present in AR and FASN immune complexes.

Progress: The progress was initially hindered by the relatively high background of co-enriched non-palmitoylated proteins and the relative low sensitivity of liquid chromatography-tandem mass spectrometry (LC-MS/MS) used in our original PalmPISC study. Thus, we systematically optimized PalmPISC and LC-MS/MS to address these limitations.

Firstly, we systematically optimized our PalmPISC method. In PalmPISC, disulfide bonds are reduced and all free cysteines are blocked by an alkylating reagent. Subsequently, palmitoyl groups are cleaved off from cysteine residues by neutral hydroxylamine, and the newly formed free thiols are conjugated to a biotin analog. Thus, the formerly palmitoylated proteins can be enriched by streptavidin affinity purification. However, we found that, with our original PalmPISC method, about 10-20% of enriched proteins are non-palmitoylated proteins. Notably, most other published methods for the enrichment of palmitoylated proteins provide much less specificity than our original PalmPISC—about 40-70% of enriched proteins were non-palmitoylated proteins.¹⁰ This relatively high background greatly hinders the identification of lowly abundant palmitoyl-proteins, whose abundance may be several orders of magnitude lower than abundant contaminant proteins. To address this issue, we systematically examined the sources for the background. We found that the background can be dramatically reduced by further blocking free cysteine residues with a commercially available compound. Using the improved procedure, co-purified contaminant proteins only account for <1% of enriched palmitoyl-proteins in amount (Fig. 1). In other words, the background was reduced by **at least 10-fold** using our new procedure.

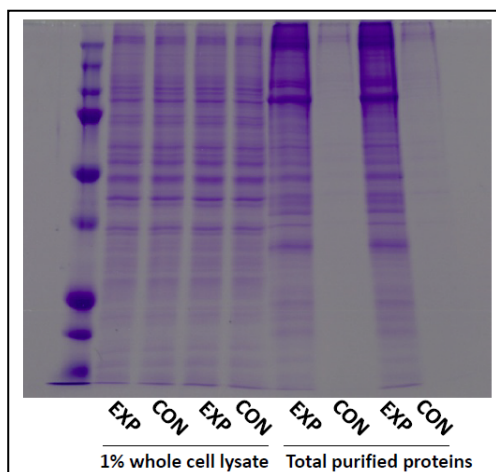


Figure 1. Over 99.9% of non-palmitoylated proteins can be depleted using our optimized PalmPISC protocol. Here, EXP indicates the experimental group, for which hydroxylamine was added to convert palmitoylated cysteines into free cysteines so that they can be specifically enriched by biotinylation of free cysteines followed by streptavidin affinity purification. CON indicates the control group, for which Tris buffer was used to replace hydroxylamine solution.

Secondly, we developed a highly sensitive LC-MS/MS method with low sample consumption. We took advantage of the LTQ Orbitrap Elite mass spectrometer installed at the Cedars-Sinai Biomarker Discovery Platform core, and systemically evaluated ten sets of LC-MS/MS settings. Consequently, we developed a workflow that enables the identification of over 5,000 proteins (with a false discovery rate of 1%) from 2 μ g LNCaP lysates in about 4 hours of LC-MS/MS analysis (Fig. 2). This represents **at least 100 times** more effective than our previous LC-MS/MS method using an LTQ Orbitrap XL.

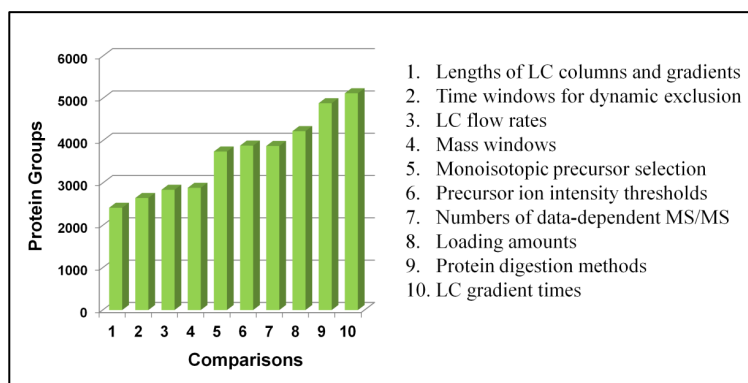


Figure 2. Development of a highly sensitive single-shot proteomics method. Systematic evaluations of 10 groups of settings led to markedly improved identification of proteins. After the optimization, we were able to identify over 5,000 proteins from merely 2 μ g LNCaP lysates, more than doubling the number of proteins identified using a method provided by the vendor Thermo Scientific.

With the drastically improved PalmPISC and LC-MS/MS methods, we are identifying palmitoylation sites of AR and analyzing palmitoyl-proteins interacting with AR and FASN. The experiments are still ongoing.

Aim 1. Task 2. Identify palmitoyl-proteins downstream from FASN using a global proteomic strategy.

Proposed plan: We will use the PalmPISC method, in concert with the SILAC method of quantitative proteomics, to analyze the entire palmitoyl-proteome in PCa cells to identify proteins that are downstream from FASN.

Progress: Previously, it was thought that only a small number of proteins are palmitoylated in human cells. However, recent compilation of palmitoyl-proteins suggested that at least 1,500 human proteins are susceptible to palmitoylation.^{11,12} Thus, before characterizing FASN-regulated palmitoyl-proteins, we conducted a deep profiling study to determine the size of the palmitoyl-proteome in PCa. By integrating our improved procedures with stable isotope labeling by amino acids in cell culture (SILAC),¹³ we identified 1,137 putative palmitoyl-proteins from LNCaP cells (FDR <0.05 and SILAC ratio > 1.5). This represents ***the largest group of palmitoyl-proteins*** identified in a single study so far. Interestingly, many candidate PCa biomarkers such as prostatic acid phosphatase, prostate-specific membrane antigen, α -methylacyl-CoA racemase, fatty acid synthase, β -2-microglobulin, and mitochondrial acetyl-CoA acetyltransferase were identified as palmitoyl-proteins. It is possible that FASN may promote PCa progression by regulating the palmitoylation of some of these candidate PCa biomarkers.

We have found that caveolin-1, a classical lipid raft protein, is required for the upregulation of FASN.¹⁴ Thus, we analyzed lipid raft-resident palmitoyl-proteins regulated by caveolin-1. We isolated lipid rafts from prostate stromal WPMY-1 cells before and after stable caveolin-1 knockdown (shCtrl vs shCav1), using our published method.¹⁵ After enriching palmitoyl-proteins, we performed label-free quantitative proteomics analysis. A total of 2,216 protein groups were identified from the purified palmitoyl protein samples (2 groups \times 3 replicates), with an FDR <0.01. As estimated by total ion intensity, the most abundant proteins are well known palmitoyl-proteins such as CKAP4, CAV1, CD44, GNAI2, STOM, DSG2, GNAI3, FLOT1 and FLOT2, confirming that palmitoyl proteins were indeed highly enriched by our method. Label-free quantification (LFQ) was performed by comparing LFQ intensities computed by MaxQuant.¹⁶ After statistical analysis, we identified 29 significantly downregulated and 32 significantly upregulated palmitoyl-proteins (Fig. 3). In addition, 187 proteins were uniquely detected in the shCav1 group while 71 proteins were only detected in the shCtrl group.

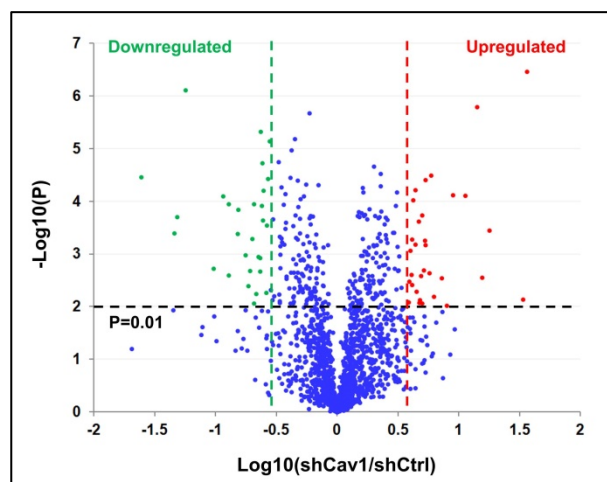


Figure 3. Volcano plot showing changes of lipid raft palmitoyl-proteins after stable knockdown of caveolin-1. For the identification of differentially expressed proteins (DEPs), two criteria were used: (1) $p < 0.01$ and (2) fold change > 3.45 . As a result, 29 proteins were identified as significantly downregulated, while 32 proteins were significantly upregulated, by caveolin-1 knockdown.

Aim 1. Task 3. Establish critical network relationships involving palmitoylated proteins.

Proposed plan: We will use bioinformatics tools and other information that arises from our proteomic studies of the palmitoyl-protein network to identify critical nodes and novel relationships between proteins within the network.

Progress: Using Ingenuity Pathway Analysis (IPA), we constructed a protein-protein interaction network for the differentially expressed palmitoyl-proteins, which include the 61 significantly changed and the 258 uniquely detected palmitoyl-proteins (Fig. 4). Interestingly, the palmitoyl-protein levels of several signaling proteins involved in PCa progression, such as Lyn, MAPK3, and RhoA, were found to increase after caveolin-1 knockdown. The validation and functional analysis of selected proteins are still ongoing.

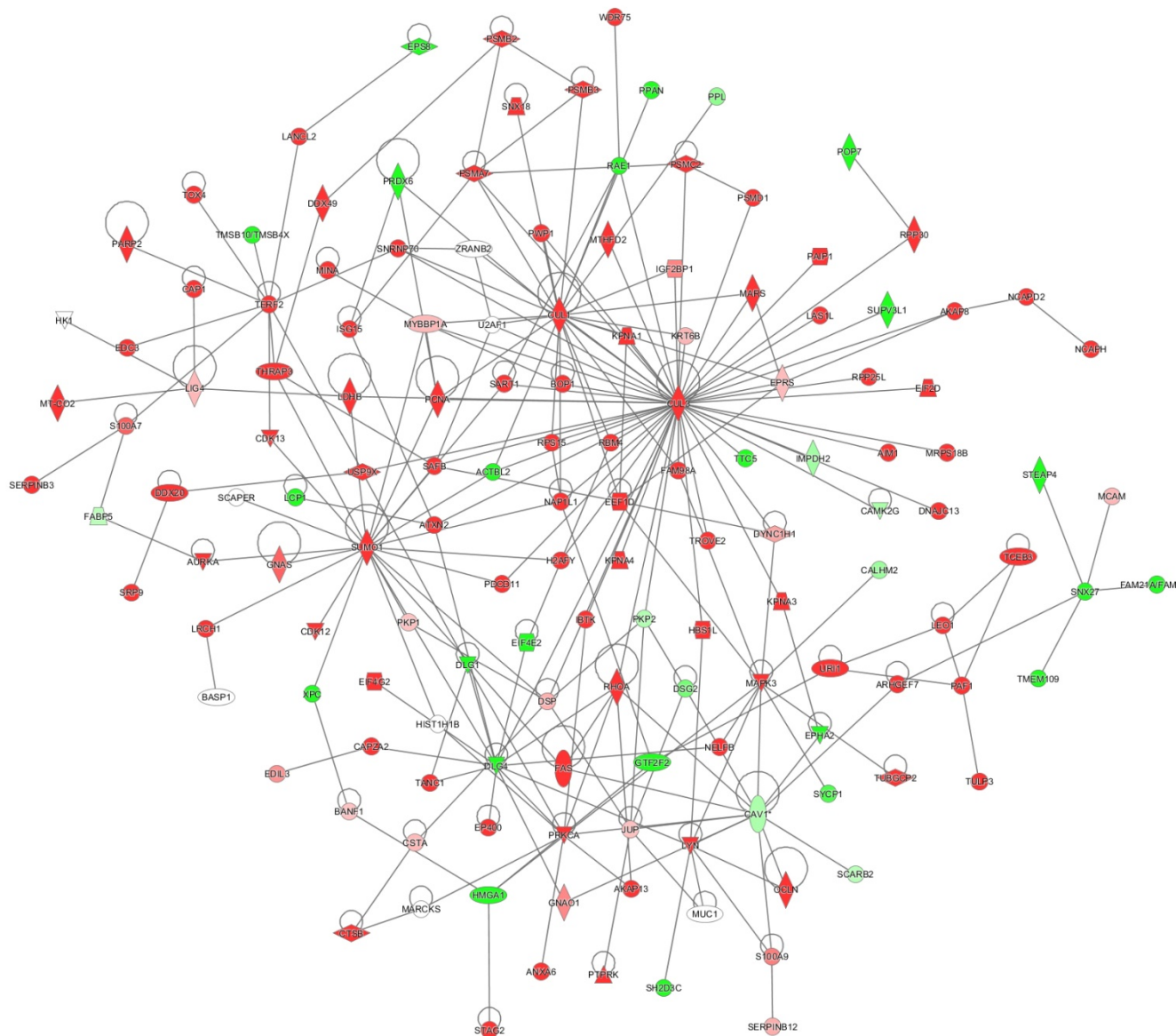


Figure 4. A virtual palmitoyl-protein complex is regulated by the knockdown of caveolin-1 in prostate stromal WPMY-1 cells. Palmitoyl-proteins that are significantly changed or uniquely detected in one group were merged, and analyzed by Ingenuity Pathway Analysis to construct the protein-protein interaction network.

Aim 1. Task 4. Test potential biological functions of critical nodes.

Proposed plan: We will use a series of functional tests to determine whether perturbation of the proteins and relationships we identify in Tasks 1-3 evoke one or more biological responses in PCa cells relevant to tumor behavior *in vivo*. We will also look for opportunities within the data set to identify substrates for one or more palmitoyl transferases (PATs), which enzymatically modify proteins with palmitate residues. In addition, we will use a series of assays developed in our laboratory to assess whether elements of the palmitoyl-protein network can be implicated in the amoeboid tumor cell phenotype, as well as secretion and activity of oncosomes, membrane-bound particles with the capability of altering the tumor microenvironment that we recently linked to metastatic PCa.

Progress: Protein palmitoylation is catalyzed by a group of Asp-His-His-Cys (DHHC) motif-containing palmitoyl acyltransferases (PATs).¹⁷ Previous studies suggested that DHHC3 plays a critical role in mediating PCa

metastasis, partly through the palmitoylation of integrin $\alpha 6\beta 4$.¹⁸ Thus, we hypothesized that some of the palmitoyl-proteins regulated by caveolin-1 may be DHHC3 substrates. To comprehensively identify candidate DHHC3 substrates, we quantitatively compared the palmitoyl-proteomes of PC-3 cells before and after stable DHHC3 knockdown (shCtrl vs shDHHC3), by integrating PalmPISC with triplex SILAC. As shown in Figure 5, three populations of PC-3 cells were metabolically labeled with isotopically different SILAC amino acids in parallel. One group of control cells were cultured in “light” medium containing natural lysine (Lys0) and arginine (Arg0), DHHC3-knockdown cells were cultured in “heavy” medium containing $^{13}\text{C}_6$, $^{15}\text{N}_2$ -lysine (Lys8) and $^{13}\text{C}_6$, $^{15}\text{N}_4$ -arginine (Arg10), and the other group of control cells were cultured in “medium” medium containing 4,4,5,5-D₄-lysine (Lys4) and $^{13}\text{C}_6$ -arginine (Arg6). After six doublings, when cellular proteins were at least 98% labeled with SILAC amino acids, control cells labeled with Lys0 and Arg0 and DHHC3-knockdown cells labeled with Lys8 and Arg10 were mixed at 1:1 ratio, and then palmitoyl proteins were isolated using our PalmPISC method. Moreover, to distinguish palmitoyl proteins from co-enriched contaminating proteins, we omitted hydroxylamine—a chemical provides selectivity for palmitoyl proteins—from our PalmPISC condition and isolated the contaminating proteins from control cells labeled with Lys4 and Arg6. Finally, we mixed the purified proteins together and performed quantitative proteomics analyses and analyzed the SILAC dataset with MaxQuant.

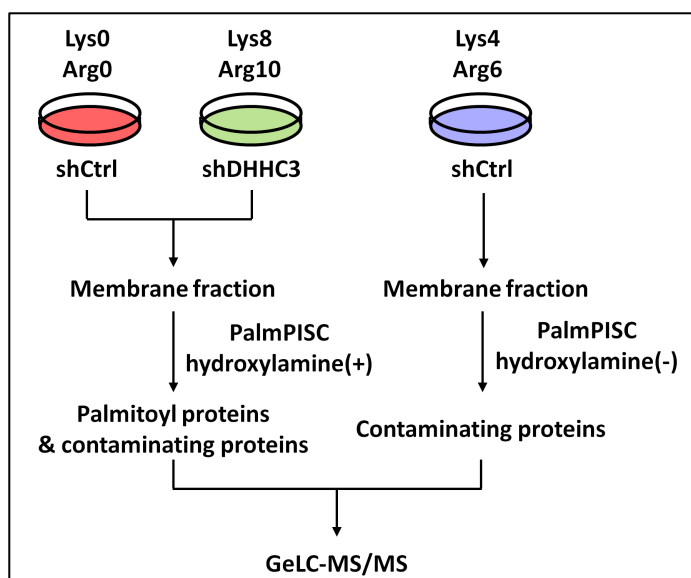


Figure 5. Workflow for the unbiased identification of DHHC3 substrates. Three populations of PC-3 cells were isotopically differentially labeled. Palmitoyl-proteins or contaminating proteins were isolated by PalmPISC, mixed, digested by trypsin, and analyzed by LC-MS/MS. Proteins were identified and quantified by MaxQuant.

Theoretically, proteins that are palmitoylated by DHHC3 (*i.e.*, DHHC3 substrates) will have a pattern of SILAC spectra shown in Figure 6A, because the knockdown of DHHC3 reduces the palmitoylation level of its substrates, whereas the omission of hydroxylamine prevents the purification of the substrates. In contrast, DHHC3 knockdown will not affect other palmitoylated proteins, thereby non-DHHC3-substrate palmitoyl-proteins will have a pattern shown in Figure 6B. In addition, contaminating proteins will have a ratio of 1:1:1 (Fig. 6C), because DHHC3 knockdown or the presence/absence of hydroxylamine will not affect their purification.

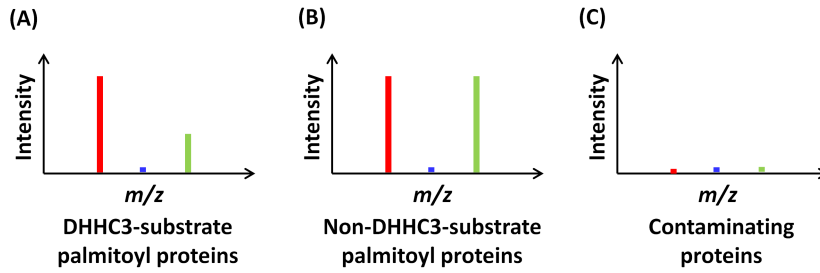


Figure 6. Theoretic patterns of SILAC spectra for (A) palmitoyl-proteins that are DHHC3 substrates, (B) palmitoyl-proteins that are not DHHC3 substrates, and (C) contaminating proteins.

A total of 529 candidate palmitoyl-proteins were identified. Figure 7A shows a representative SILAC spectrum of a peptide derived from cytoskeleton-associated protein 4 (CKAP4), a known palmitoyl protein.¹⁹ DHHC3 knockdown led to the decrease of the palmitoylation level of CKAP4, suggesting that CKAP4 is a candidate substrate of DHHC3. In contrast, as shown in Figure 7B, the palmitoylation level of flotillin-1 (FLOT1), also a known palmitoyl-protein,²⁰ was not affected by DHHC3 knockdown, indicating that flotillin-1 is unlikely a DHHC3 substrate.

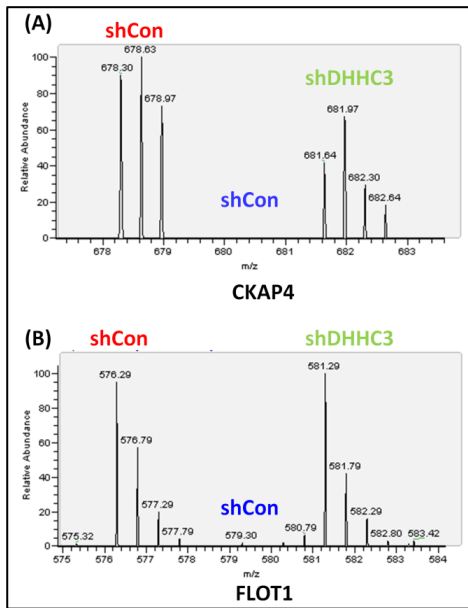


Figure 7: Representative SILAC spectrum of a peptide derived from (A) a candidate DHHC3 substrate cytoskeleton-associated protein 4 (CKAP4) and (B) an unlikely DHHC3 substrate flotillin-1 (FLOT1).

After statistical analysis, 30 candidate palmitoyl-proteins with an H/L ratio cutoff of 0.667 were accepted as candidate DHHC3 substrates (Table 1). Among these, the most dramatically downregulated protein is the DHHC3 protein itself. In addition, the candidate substrates include integrin subunits $\alpha 6$ and $\beta 4$, which have previously been shown to be DHHC3 substrates, corroborating the effectiveness of our triplex SILAC-PalmPISC approach.¹⁸ Interestingly, Tumor necrosis factor receptor superfamily member 6 (FAS), a key node protein of the Cav1-downstream palmitoyl-protein network (Fig. 4), is a candidate DHHC3 substrate. A further functional analysis of palmitoylated FAS is currently ongoing.

Table 1. Candidate DHHC3 substrates in prostate cancer PC-3 cells

Protein Description	Gene	Ratio H/L
Palmitoyl acyltransferase DHHC3	ZDHHC3	0.244
Linker for activation of T-cells family member 2	LAT2	0.324
Chromobox protein homolog 5	CBX5	0.337
Hephaestin-like protein 1	HEPHL1	0.356
Anoctamin-1	ANO1	0.395
Integrin alpha-6	ITGA6	0.407
Transmembrane protein 192	TMEM192	0.446
Transmembrane emp24 domain-containing protein 1	TMED1	0.459
CD58	CD58	0.485
Cytoskeleton-associated protein 4	CKAP4	0.490
CKLF-like MARVEL transmembrane domain-containing protein 6	CMTM6	0.510
Beta-1,4-galactosyltransferase 1	B4GALT1	0.515
Tetraspanin-9	TSPAN9	0.515
Transmembrane protein 97	TMEM97	0.546
24-dehydrocholesterol reductase	DHCR24	0.549
B-cell receptor-associated protein 29	BCAP29	0.559
Cytochrome b	MT-CYB	0.559
Protein disulfide-isomerase TMX3	TMX3	0.571
Carboxypeptidase D	CPD	0.585
Integrin beta-4	ITGB4	0.585
Surfeit locus protein 4	SURF4	0.588
CD44 antigen	CD44	0.599
Tumor necrosis factor receptor superfamily, member 6 isoform 1 variant	FAS	0.617
Guanine nucleotide-binding protein subunit beta-2-like 1	GNB2L1	0.637
Endoplasmic reticulum-Golgi intermediate compartment protein 2	ERGIC2	0.645
Cation-dependent mannose-6-phosphate receptor	M6PR	0.645
N-acylsphingosine amidohydrolase 1	ASAH1	0.658
Disintegrin and metalloproteinase domain-containing protein 17	ADAM17	0.658
Protein jagged-1	JAG1	0.667
40S ribosomal protein S27a	RPS27A	0.667

We recently showed that large oncosomes, a type of extracellular microvesicles with a diameter of $> 1 \mu\text{m}$, are a feature of metastatic PCa.²¹ We hypothesized that some palmitoyl-proteins are contained in large oncosomes secreted by PCa cells and that the palmitoyl-proteome in large oncosomes is different from that in exosomes. Thus, we performed a SILAC comparison of large oncosomes and exosomes secreted from PCa DU-145 cells. Consistent with our hypothesis, some palmitoyl-proteins (*e.g.*, STOML2) are enriched in, whereas some (*e.g.*, CD44, CD9 and CD81) are depleted from, large oncosomes (Fig. 8). We are currently investigating whether elements of the palmitoyl-protein network are implicated in the amoeboid tumor cell phenotype as well as secretion and activity of oncosomes.

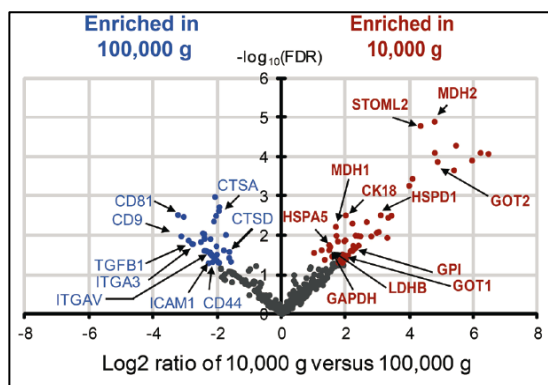


Figure 8. Volcano plots of the log2-transformed SILAC ratios against the false discovery rate (FDR). Red dots correspond to proteins enriched in large oncosomes harvested by 10,000×g ultracentrifugation; blue dots correspond to proteins enriched in small extracellular vesicles isolated by 100,000×g ultracentrifugation.

Specific Aim 2: Determine whether the FASN-palmitoyl network can be suppressed *in vivo* by cholesterol reduction.

Altered cholesterol levels changed bone metastasis of prostate cancer cells.

Collaborating with Dr. Leland Chung's group at Cedars-Sinai, we confirmed the aggressive and the indolent PC using experimental mouse models subjected to androgen-deprivation therapy or exposed to high cholesterol diet. In this study, we showed intracardiac injected LN-RANKL cells were metastasized more prevalently to bone and soft tissues in androgen-deprived or high cholesterol-fed mice. These behavioral differences of metastases were confirmed by CTC numbers as well as the criteria established above in distinguishing aggressive versus indolent PC based on their RANKL/CK13 status.

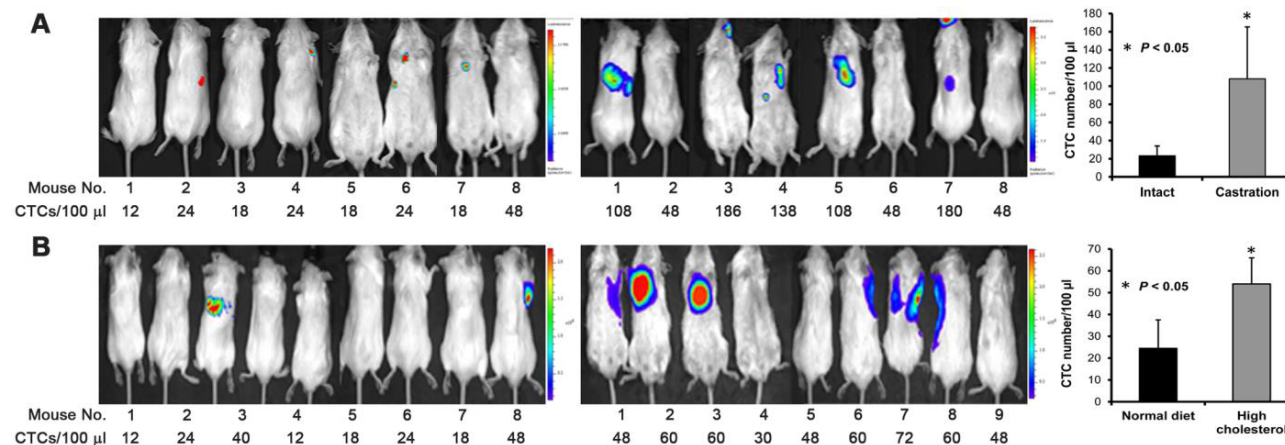


Figure 8. Androgen deprivation and high cholesterol diet enhanced PC bone and soft tissue metastases. A, Mice (N=8 per group) were either sham operated (Intact) or surgically castrated (Castrated) prior to i.c injection of LNRANKL-Luc-RFP cells. Mice were imaged every two weeks and their CTC counts were obtained on the cytospin slides. Note higher number of metastasis, detected by bioluminescence, accompanied by higher number of CTCs in castrated than sham operated intact mice at 16 weeks. B, Similar study were conducted in mice fed with normal or high cholesterol diet. Note higher number of metastasis, detected by bioluminescence, accompanied by higher number of CTCs in high cholesterol diet fed than normal diet fed mice at 12 weeks.

Other *in vivo* experiments testing the role of ezetimibe in suppressing PCa tumor growth are ongoing.

Key research Accomplishments

- Improved the selectivity of purifying palmitoylated proteins by >10 times.
- Developed a highly sensitive LC-MS/MS method by systematically evaluating 10 sets of LC-MS/MS settings. The new method is >100 times more effective than our previous method.
- Conducted an in-depth palmitoyl-proteomics profiling study of LNCaP cells and identified >1,000 palmitoyl-proteins.
- Identified a palmitoyl-protein network downstream of caveolin-1.
- Identified candidate DHHC3 substrates in prostate cancer PC3 cells.
- Identified proteins significantly enriched in large oncosomes.
- Found that high cholesterol diet enhanced PCa bone and soft tissue metastases.

Conclusion

By substantially improving the selectivity of our PalmPISC method as well as the speed and sensitivity of LC-MS/MS analysis, we developed a very powerful palmitoyl-proteomics profiling method. By coupling the method with duplex SILAC, we conducted so far the most comprehensive palmitoyl-proteome profiling study, and identified 1,137 candidate palmitoyl-proteins from LNCaP cells. Using label-free quantitative palmitoyl-proteomics, we identified a gigantic palmitoyl-protein network regulated by caveolin-1. Moreover, by integrating RNA interference (RNAi), triplex SILAC, and PalmPISC, we identified 30 DHHC3 substrate candidates in prostate cancer PC3 cells, among which FAS is a key node protein of the Cav1-downstream palmitoyl-protein network. In addition, we identified proteins enriched in or depleted from DU145-secreted large oncosomes, compared with exosomes.

Publications, Abstracts, and Presentations

1. Zhou, B., An, M., Freeman, M. R., and Yang, W. Technologies and challenges in proteomic analysis of protein S-acylation. *J. Proteomics Bioinform.* 2014, 7: 256-263.
2. Minciaccchi, V.R., You, S., Spinelli, C. et al. Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles. *Oncotarget.* 2015, 6: 11327-41.
3. Zhou, B., Freeman, M. R., and Yang, W. Single-shot proteomics profiling and quantification of human prostate cancer cells. Abstract for the 14th Human Proteome Organization (HUPO) World Congress (2015, Vancouver, Canada)

Inventions, Patents and Licenses

N/A

Reportable Outcomes

- A markedly improved method for purification of palmitoyl proteins with high (>99.9%) selectivity.
- A powerful LC-MS/MS analysis method for highly sensitive identification of proteins from micrograms of proteins.
- The largest group of palmitoyl proteins identified in a single study so far.
- The lipid raft-resident palmitoyl-protein network regulated by caveolin-1.
- DHHC3 substrates identified from PC-3 cells.
- Cell motion is the most significantly regulated biological process by DHHC3 in cancer.

Other Achievements

DoD W81XWH-15-1-0167

Yang (PI)

06/15/15-06/14/16

DHHC2, a Palmitoylating Enzyme, is a Key Suppressor of Prostate Cancer Metastasis and Castration Resistance
The objective of this project is to test the overall hypothesis that DHHC2 suppresses prostate cancer metastasis and castration resistance via the palmitoylation of certain substrates critical for prostate cancer progression to metastatic castration-resistant prostate cancer.

DoD PC141605

Sobreiro (PI)

09/01/15-08/31/17

Does the Loss of Stromal Caveolin-1 Remodel the Tumor Microenvironment by Activating Src-mediated PEAK1 and PI3K Pathways?

The objective of the proposed project is to test the hypothesis that the loss of caveolin-1 in prostate stroma may remodel the tumor microenvironment and thus facilitate prostate cancer progression, at least in part, by degrading the extracellular matrix and by regulating the secretion of certain extracellular molecules, either in water-soluble or extracellular vesicle-confined form.

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Appendices

N/A